DEVELOPMENT OF PIGMENT IN THE LARVA OF THE SEA URCHIN, LYTECHINUS VARIEGATUS[1, 2]

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Very little is known concerning the origin of invertebrate pigment cells. Most invertebrate eggs contain varying amounts of carotenoid pigments, which are sufficient to mask the appearance of any new pigments. The unsegmented egg of the sea urchin *Lytechinus variegatus,* however, contains very small amounts of carotenoid or other pigment material and formation of a new pigment (echinochrome) in the early gastrula stage is readily observed. The striking appearance of pigment in early development suggests that the eggs of this animal might be unusually favorable material for a study of the cellular origin and chemo-differentiation of a defined substance—echinochrome. Echinochrome is a substituted naphthoquinone, red-purple in color, found in the test, spines, epidermis and various internal organs of sea urchins. The chemical structure and physical and chemical properties of certain echinochromes have been established by various investigators (Ball, 1936; Lederer and Glaser, 1938; Glaser and Lederer, 1939; Kuhn and Wallenfels, 1939, 1940; Wallenfels and Gauhe, 1943; Goodwin and Srisukh, 1950). A number of physiological functions have been ascribed to echinochrome. However, questions concerning the embryological and biochemical derivation, metabolism, and possible physiological functions of polyhydroxynaphthoquinones in echinoids are mainly unanswered.

The present study is an attempt to determine the embryonic origin of the echinochrome-forming cells, and to throw some light on the intracellular mechanisms affecting echinochrome synthesis.

Materials and Methods

The eggs and sperm of the sea urchin *Lytechinus variegatus* were used throughout this work. *Arbacia punctulata* was used in some instances for comparison. The animals were collected in the Gulf of Mexico at the mouth of Alligator Harbor, Florida, using the facilities of the Florida State University Marine Laboratory. The animals were maintained in running sea water or jugs of continually aerated sea water at about 15 degrees centigrade.

The eggs and sperm were obtained from the animals by the KCl injection method. Eggs were fertilized in filtered sea water with approximately 0.5% sperm suspensions.

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Development was observed with the dissecting microscope and the phase contrast microscope. The ultra violet absorption spectrum of the echinochrome was determined by means of the Beckman DU Spectrophotometer.

All results are based on experiments involving at least 200 eggs. Each experiment was repeated at least twice. Any deviation from these numbers will be discussed in the text. The various techniques used in the individual experiments will be described with the results of the experiments for the sake of continuity and clarity.

Results

Normal development

The fertilized egg of *Lytechinus* is relatively pigment-free. There is a very faint yellowish cast to the egg, probably due to carotenoids as in most echinoderm eggs, but the nucleus and cellular inclusions are clearly visible under the dissecting microscope.

The echinochrome-containing cells (echinophores) are first noticeable under the microscope during early gastrulation, when the veg_2 cell layer is invaginating to form the gut. They appear to be in the ectodermal layer and at first only in the region of the invagination. As gastrulation proceeds, the echinophores become dispersed throughout the gastrula, apparently in the ectodermal layer. In the pluteus, these cells are evenly dispersed in the outer body wall, with usually some concentration in the arm tips.

A series of experiments was designed to determine more precisely the origin of these cells in the course of development of the embryo and perhaps what intra- or inter-cellular mechanisms were involved.

Lithium experiments

Exogastrulae were produced using the technique of Herbst (1892) (treatment of fertilized eggs in a 0.1 *M* LiCl solution in sea water for five hours). This presented an opportunity to determine the effect of LiCl on pigmentation and to determine if the normal association of the germ layers is necessary for pigmentation. These exogastrulae were striking in that echinophores always appeared in the ectodermal portion of the larvae and never in the endoderm. The pigment appeared first at the site of evagination and by the time the evaginated gut was completely formed these cells were dispersed throughout the ectodermal wall of the larvae. The evaginated gut wall never contained pigment cells, although occasionally pigment could be seen inside the lumen where apparently it had migrated. This particular phenomenon will be discussed in more detail later, in connection with the amoeboid movements of these cells. From this experiment it may be said that pigment is associated with the ectoderm (at least in exogastrulae) and that a normal association of the germ layers is not necessary for pigment formation. LiCl in the concentrations (0.1 *M*–0.5 *M*) used had no apparent effect on pigmentation.

Thiocyanate experiments

Since the pigment cells were found only in the ectodermal portions of the LiCl-induced exogastrulae, it would be interesting to know if the ectoderm alone is able to give rise to these echinophores.

"Dauer" blastulae (permanent blastulae with no gut or skeleton) were formed using NaSCN (0.125 *M*) and the technique of Lindahl (1936). Those embryos which developed into "Dauer" blastulae were never pigmented. They remained as colorless hollow balls of ciliated ectodermal cells, whereas if there was any sign of invagination and appearance of endodermal derivatives, pigment was always formed in the ectodermal regions. In these cases, where very little endoderm was present, pigment cells were few and widely scattered. However, as in previous experiments, the pigment first appeared around the invaginating region, and later gradually became dispersed throughout the larval wall.

Apparently the ectoderm alone is not capable of giving rise to pigment, at least not in those "Dauer" blastulae formed by treatment with NaSCN.

Isolation experiments

It is possible to study the effects of ectodermization on pigmentation, without the influence of a chemical agent such as NaSCN. In these experiments, the technique of Hörstadius (1928) was used. The most satisfactory method for removing the fertilization membranes was found to be the shaking method of Driesch (1891).

Since *Lytechinus* eggs have no pigment band and removal of the fertilization membrane also removes the polar bodies at the animal pole, there is no sure way of determining which is the animal and which is the vegetal pole at the 8-cell stage. However, at the 16-cell stage, the micromeres have appeared and identification of the vegetal pole is quite easy. For this reason, most of the operations were done at the 16-cell stage. Some, however, were done at the 8-cell stage and will be discussed later. About 10 eggs at the 16-cell stage were placed in a drop of sea water in the shallow depression in the lid of a small stender dish, by means of a capillary pipette. The drop of sea water was kept as small as possible so that the surface tension served to hold the embryos in place while the cuts were being made. Under the dissecting microscope the desired blastomeres were separated with glass dissecting needles. The separated cells were picked up with the capillary pipette, transferred to sea water in syracuse dishes and allowed to develop.

By means of this technique, 30 animal and 30 vegetal halves were isolated at the 16-cell stage. Invariably, the animal halves formed typical "Dauer" blastulae, while the vegetal halves gastrulated and sometimes formed miniature plutei. These were usually defective in that they often had only one arm or a poorly formed gut and skeleton. The "Dauer" blastulae from isolated animal halves were never pigmented and remained unpigmented until they died. The vegetal half-embryos were always pigmented in a typical fashion.

Similar results were obtained in 40 cases in which halves of the 8-cell stage were isolated. However, since there was no way of identifying the animal and vegetal halves, the assumption was made that some of the cuts would isolate halves containing two animal and two vegetal cells, depending on the plane of the cut. This assumption appeared to be well founded, since some halves formed "Dauer"

blastulae, while most developed into pigmented plutei. Only the animal halves form "Dauer" blastulae whereas the vegetal halves and those halves containing two animal and two vegetal cells gastrulate and develop pigment.

These experiments confirmed the results obtained in the NaSCN experiments, that is, that the ectoderm alone does not produce pigment. Pigment is formed when the embryo gastrulates (regardless of what fraction of the original egg is present) and only if the embryo gastrulates.

Vital staining experiments

In order to determine exactly what cells are responsible for the production of these echinophores, the technique of vital staining was employed. The technique of Hörstadius (1935) was used. The dye used was Grüblers "Neutral Rot," since this was the only dye found to penetrate the larvae satisfactorily.

The eggs were stained at the 16-cell stage. A fine glass capillary which was filled with agar containing a one per cent solution of neutral red was put into a drop of sea water in a stender dish lid. The egg to be stained was moved into position and held in place for about one minute, at which time enough dye was absorbed to render it readily identifiable. At the 16-cell stage the eight animal cells were stained in this way in a series of 12 eggs. These stained cells never became pigmented after gastrulation. The four micromeres in 12 eggs were stained in the same way and here, too, the stained cells never produced echinophores. When the four macromeres (of 12 eggs) were stained, the pigment cells in the pluteus showed traces of the dye. The macromeres do not divide until the end of the 32-cell stage, so that staining of the veg_1 and veg_2 cells derived from the macromeres is impossible until the 64-cell stage. At the time of the 64-cell stage, the individual blastomeres are extremely small and difficult to stain individually. However, an attempt was made to stain the cells comprising veg_1 in one series of eight eggs, and veg_2 in another series of eight eggs. It was found that the veg_2 cells and the micromere material always invaginated at gastrulation and gave rise to no pigmented cells. Although it was almost impossible to stain only veg_1 cells since some of the stain invariably got into veg_2 cells, it was none the less possible to see that it was from this material that the echinophores ultimately arose, since the concentration of the dye in the veg_1 cells was much greater. The material from veg_1 did not invaginate, but at the time of gastrulation, it remained near the site of invagination and ultimately gave rise to the ventral ectoderm of the pluteus.

Therefore, it would seem that the echinophores originate from the veg_1 cells but only under conditions permitting axial differentiation. The question then arises as to how these pigment cells become dispersed throughout the ectoderm of the pluteus, if their origin is localized in material which is ultimately destined to become only the ventral ectoderm of the pluteus. A series of observations gave an answer to this question.

Phase microscope observations

Boveri (1901) noticed amoeboid cells, of a brick red color, appearing in the late gastrula stage of the sea urchin *Paracentrotus lividus*. He considered the

pigment the same as that in the egg. Monroy *et al.* (1951) also noted these amoeboid cells and suggested the possibility that the pigment in them might be echinochrome, although they were not able to obtain enough material to prove its presence. Neither of these workers reported a detailed study of the movements of the pigment cells, or their location.

These cells were studied in *Lytechinus variegatus* under the phase contrast microscope. In the early gastrulae when the echinophores first appeared, it was found that the cells were large, irregularly shaped structures in the region of the invagination. This would be the material derived from veg_1 cells. First, small pale orange pigment granules appeared. These were not easily visible except under high magnification. After a few hours, when the invagination process was almost complete, the pigment in these cells became darker red in color and much more concentrated, while the cells themselves increased greatly in number. At this time, it could be seen that the pigment cells were amoeboid in nature, apparently able to move freely within the ectodermal layer in any dimension. However, they were never seen to enter the endodermal layer underneath. By the pluteus stage, they had invaded the ectoderm of the larva and appeared to be most concentrated in the more actively growing arm tips of the pluteus. By the time the pluteus was fully formed, the echinophores had become much less motile and migration had virtually ceased.

In observations of eight exogastrulae, the same pattern was seen, except that the endoderm was no longer immediately under the ectoderm. The echinophores were seen occasionally to work themselves completely free of the ectoderm and come to lie in the cavity of the blastocoel and eventually even in the everted gut lumen. This explains the occasional appearance of pigment in the gut cavity of exogastrulae.

The next series of experiments was designed to determine what physical and chemical factors in the developing egg were responsible for the formation of the pigment and to obtain some information as to the relative roles played by the nucleus and cytoplasm in this synthesis. Since the pigment is elaborated long before the organism begins to take in food material from the outside, the pigment must be synthesized from pre-existing materials present in either the egg or sperm, or both.

Hybridization experiments

A series of experiments was designed to determine the effect of *Arbacia* × *Lytechinus* hybridization on subsequent pigmentation. Tennents' (1912) method (ageing of gametes for two hours followed by a five-minute treatment with alkaline sea water before fertilization) was used in obtaining these hybrids. The larvae from *Arbacia* male × *Lytechinus* female were all maternal in appearance. Pigmentation, size and general body structure of gastrulae (very few reached the pluteus stage) were typical of *Lytechinus*. The reverse cross, *Lytechinus* sperm × *Arbacia* egg, was unsuccessful in that no gastrulae were obtained and nothing could be seen concerning any newly formed pigment cells at gastrulation. These experiments were repeated three times with practically identical results.

It can be said only that *Arbacia* sperm cannot affect normal pigmentation in the

Lytechinus egg, in *Arbacia* sperm × *Lytechinus* egg hybrids. The question then arises as to the normal role, if any, played by the sperm in pigmentation.

One way of answering this question is to study the development of artificially activated eggs.

Parthenogenesis experiments

The (butyric acid-hypertonic sea water) method of Tennent (1912) was found to be the most satisfactory for producing parthenogenetic larvae. Again, it was noted that if gastrulation occurred, pigment was formed and apparently in the same fashion as described for normal fertilized eggs. The parthenogenetic plutei were normally pigmented and quite like the fertilized controls. These experiments were also repeated three times with the same results. It would seem that the presence of the sperm cell is not essential for pigment formation.

Identification of the pigment

The assumption has been made so far that the pigment in question is the substituted naphthoquinone, echinochrome. The proof of this lies in isolation, physical and chemical properties and spectrophotometric analysis. Echinochrome may be extracted by treatment with slightly acidified, organic solvents such as 80 per cent acetone or ether containing one per cent of HCl. The pigment may be then transferred by dilution into diethyl ether and chromatogrammed to remove impurities. The free compound shows very slight solubility in water or petroleum ether but is readily restored by shaking in air or by any of a number of mild oxidizing agents (Ball, 1936).

Clearly defined absorption bands are exhibited by solutions of echinochrome in various solvents. According to Kuhn and Wallenfels (1939), the absorption maxima of an echinochrome solution in carbon disulphide were 535, 499 and 464 mμ, in chloroform 532, 497 and 462 mμ, in benzene 532, 494 and 461 mμ and in concentrated sulfuric acid, 502 and 469 mμ.

The following evidence shows that the pigment appearing at gastrulation in the sea urchin, *Lytechinus variegatus,* has the properties of echinochrome.

(1) The pigment is orange-red in color.
(2) The pigment may be extracted from gastrulae and plutei by the above described procedure.
(3) The pigment, when extracted and dried, is nearly insoluble in water and petroleum ether.
(4) The pigment turns red in acid solution and violet in the alkaline range.
(5) The pigment is soluble in diethyl ether, acetone, ethanol and carbon disulfide.
(6) The pigment is very slightly soluble in chloroform.
(7) The addition of 5 mg. of sodium hydrosulfite to 10 ml. of a brick red solution of the pigment quickly bleaches the solution.
(8) The addition of small amounts of an oxidizing compound (hydrogen peroxide) or shaking in air quickly restores the color to the solution.
(9) The absorption spectrum of a carbon disulfide-pigment solution showed peaks on the Beckman DU Spectrophotometer at 530, 491, and 460 mμ.

Carotenoids, chromolipids, melanins and flavins may also be reddish in color (Sumner and Doudoroff, 1943). However, melanins and flavins (Mayer and Cook, 1943) are insoluble in almost all organic solvents. Flavins are water-soluble. The carotenoids may be bleached (Fox, 1936) but only by oxidizing agents rather than reducing agents. The absorption spectrum is typical of echinochrome and not carotenoids, since the carotenoid absorption peaks are around 510 and 485 mμ (Fox and Scheer, 1941). The change in color with changes in pH is also typical of quinone pigments. It was not possible to isolate and crystallize a sufficient amount of the pigment from plutei to permit further analysis of its physical and chemical properties but on the basis of the above evidence, the pigment appears to be echinochrome.

Chemo-differentiation study

The effect of a large number of inhibitors was studied in an attempt to specifically inhibit pigmentation and perhaps learn somethong of the metabolic pathway involved in its synthesis. It was found, however, that only those inhibitors which stopped development at gastrulation or ectodermized the eggs, such as 2-4-dinitrophenol, pyocyanine and iodosobenzoic acid, had an effect on pigmentation.

Discussion

Boveri (1901) first noticed in the late gastrula stage of the sea urchin *Paracentrotus lividus* the appearance of amoeboid cells, rather heavily loaded with large pigment granules which differed from those of the unsegmented egg both with respect to their larger size and to their color. He noted that the number of these cells increased rapidly with the age of the embryo. Boveri, however, considered the pigment of the same nature as that of the egg. Monroy *et al.* (1951) noted that starting from the stage when the new pigment appeared, the embryos still retained a red-violet color after having been exhaustively extracted for carotenoids and that the remaining color was due to pigment still present in the amoeboid cells. These workers showed that the pigment could not be extracted with chloroform, acetone, methanol or pyridine but on slight acidification with dilute HCl, it could be taken up quantitatively in ether. It was thought the pigment was probably echinochrome but they were not able to obtain sufficient amounts to prove it spectrophotometrically. They also noted that the eggs of one female developed normally up to the beginning of gastrulation when exogastrulation occurred. In this case, they were unable to detect echinochrome. This observation seems unlikely in view of the exogastrulation experiments of the present study and was probably due to masking by other pigment.

Gustafson and Lenique (1951), using *Psammechinus miliaris,* mentioned pigment formation in the gastrula stage. They did not identify the pigment. However, they did mention that the red pigment cells became especially concentrated in the arm tips and the apical region, where the ectoderm is characterized by high mitochondrial activity. This observation is confirmed in the present study as previously mentioned, where echinophores are concentrated in the arm tips and apical region. It was also noted that in advanced starving plutei, the amount of echinochrome is appreciably reduced, suggesting the use of this protein-echinochrome complex as a food source under extreme conditions.

Using the *Lytechinus* egg in which the pigment is not sufficient to mask pigment elaboration subsequent to fertilization, it is possible to trace the differentiation of chromatophores with considerable accuracy. It was found that a pigment was synthesized in the embryo in the gastrula stage. This pigment was echinochrome. The particular cells in which the pigment appeared were shown by vital staining to originate from veg_1 and to be amoeboid in nature. With the use of isolation techniques and chemical treatment it was shown that pigment formation occurred only in association with gastrulation. Evidently, pigment cell differentiation is related to gastrulation in some way. Since the pigment cells differentiate in exogastrulae as well as in normal embryos, the differentiation does not appear to be an induction effect, at least to the extent that it requires the juxtaposition of endoderm with the other germ layers during gastrulation. It appears more likely that pigment cell differentiation, including the formation of pigment itself, is under the same control system as that governing the differentiation of other parts of the embryo (*e.g.,* skeleton, muscle, gut, coelom, etc.). From the work of Runnström (1933) and especially Hörstadius (1939) this over-all differentiation appears to depend upon the quantitative interaction of some sort of double gradient system. This system may function to produce pigment cells from veg_1 in normal development, but when the system is modified experimentally, for example by surgical or chemical treatments that result in "Dauer" blastulae, then pigment cells as well as other types of tissue fail to differentiate.

The production of echinochrome by the differentiating chromatophores presents an interesting problem in the chemo-differentiation of a defined substance. Unfortunately, no information is available concerning the pathways of echinochrome synthesis. However, echinochrome production may be correlated with protein synthesis, at least to the extent that new enzymes required for echinochrome synthesis may be elaborated by the embryo. Furthermore it is known that echinochrome occurs in the form of a protein complex (Kuhn and Wallenfels, 1940; Shapiro, 1946), and extensive protein synthesis begins at the same time that echinochrome first appears in the embryo, namely, at the time of gastrulation (Caspersson, 1947; Brachet, 1941; Zeuthen, 1951; Hultin, 1950; Perlmann, 1954). It is of interest to note the similarities between melanophore development in the vertebrates and echinophore development in the sea urchin. DuShane (1935) proved the neural crest origin of pigment cells in the amphibian. The formation of the neural crest and subsequent pigmentation are dependent on gastrulation in the amphibian and both amphibian and *Lytechinus* pigment cells are ectodermal in origin. In the amphibian, however, pigment cell formation is more complex, in that gastrulation induces the formation and differentiation of the neural crest, which in turn differentiates still further, giving rise to a number of structures, among which are the pigment cells. These cells, too, are amoeboid and migrate to their definitive position (Twitty and Niu, 1954) where they apparently lose their amoeboid capabilities and come to rest.

Summary

1. A pigment having the properties of echinochrome is synthesized in the embryo of the sea urchin *Lytechinus variegatus*. Differentiation of the echinophores and synthesis of the echinochrome begins at the gastrula stage.

2. Echinophores differentiate from the veg_1 cell layer of the embryo, become amoeboid and migrate into other ectodermal regions.

3. Echinophore differentiation appears to depend upon a normal relation of the "double gradient" system of the embryo. Since echinophores were produced in exogastrulae, normal juxtaposition of the germ layers is not essential.

4. The sperm nucleus was found to have no essential role in the pigmentation process. Pigment formed according to the maternal pattern in hybrid and parthenogenetic embryos.

5. Of a variety of chemical substances tested, including several respiratory and other inhibitors, only those agents which inhibited gastrulation of the embryo caused failure of pigment formation.

6. Echinochrome synthesis is apparently related to protein synthesis in the embryo.

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